FEBS Letters 578 (2004) 116–120 FEBS 29015

Organs from mice deleted for NRH:quinone oxidoreductase 2 are deprived of the melatonin binding site MT_3

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Received 26 September 2004; accepted 20 October 2004

Available online 11 November 2004

Edited by Jesus Avila

Abstract Two melatonin receptors (MT_1 and MT_2) have been cloned. A third melatonin binding site, MT_3, is known with remarkable and distinct pharmacological properties. We previously reported the purification of MT_3 and identified it as the enzyme dihydronicotinamide riboside:quinone reductase 2 (NQO2). To investigate the relationship between NQO2 and MT_3, we generated a NQO2$^{-/-}$ mouse strain. These mice no longer present MT_3 binding sites as measured with 2-[^{125}I]-iodo, 5-methoxycarbonylamino-*N*-acetyltryptamine, the specific MT_3 radioligand. These data establish NQO2 as part of the MT_3 binding sites in vivo and resolve the matter of the nature of the third melatonin binding site.

Keywords: Melatonin receptor; MT3; Dihydronicotinamide riboside:quinone oxidoreductase 2; Knock-out mice; Quinone reductase 1

1. Introduction

Melatonin is a serotonin-derived neurohormone produced in the pineal gland that relays information about the photoperiod to the peripheral organs for daily and seasonal physiological regulations [1–3]. The cellular targets of melatonin have been detected by binding studies with a specific agonist, 2-iodomelatonin, in numerous peripheral tissues of birds and mammals [4], as well as in lower organisms, including plants [5]. Three melatonin receptors have been cloned to date. Two melatonin receptors (MT_1 and MT_2) have been extensively studied [6–10]. Another one, not expressed in mammals [11], the Mel1c, was cloned from *Xenopus laevis* [12]. In mammals, a third binding site has been described, MT_3, initially in brain and kidney from hamster [13] and then in different species [14–17]. It has remarkably different pharmacological properties [13–17] distinct from those of MT_1 and MT_2. These receptors share a common seven-transmembrane predicted structure and the ability to transduce extra-cellular signals via G-protein coupling [18,19]. These two receptors also share close pharmacological profiles [8,10], including subnanomolar affinities for melatonin and 2-iodo-melatonin.

The ligands of MT_3 are specific over MT_1 and MT_2 and include to date 2-methoxycarbonylamino-*N*-acetyltryptamine (MCA-NAT) [13], nitroindole derivatives [20] and prazosin. Accordingly, an original ligand developed in the past for MT_3 studies, [^{125}I]-2-iodo, 5-methoxycarbonylamino-*N*-acetyltryptamine (2-[^{125}I]-MCANAT) [16], was preferentially used in the present work. Besides its original pharmacology, MT_3 displays very fast kinetics of ligand association/dissociation, which made affinity measurements difficult [16,17]. We purified this protein to homogeneity, sequenced it by mass spectrometry [21] and identified it as dihydronicotinamide riboside (NRH):quinone oxidoreductase 2 (NQO2), an analog of the detoxification process machinery enzyme, quinone reductase 1 (QR1) [22,23]. The expression of NQO2 in naïve cells led to the MT_3 pharmacology [21]. Nevertheless, despite this demonstration, other studies were needed to unambiguously demonstrate that this enzyme represented all the MT_3 melatonin binding sites. Among them, the construction of a mouse strain invalidated for NQO2 gene was an essential tool for the understanding of this receptor. In the present work, we report that the organs of mice deleted for NQO2 are depleted of MT_3 binding sites.

2. Materials and methods

2.1. Construction of the targeting vector

Genomic clones containing the murine NQO2 locus were isolated from a 129Sv/J-BAC genomic library (Incyte Genomics) by using probe corresponding to the murine NQO2 exon 1. The genomic organization of the locus was determined by subcloning *Pst*I and *Sac*I genomic fragments into the pZErO™-2 vector (Invitrogen, Carlsbad, USA). The 16-kb *Pst*I genomic insert and 4.8-kb *Sac*I genomic insert were sequenced and mNQO2 sequence was generated. Both genomic clones (containing promoter and exon 1 to intron III) were used to construct the targeting vector. Briefly, a 7.6-kb *Sna*BI/*Bst*EII fragment that includes exons 1–3 and a 1.4-kb *Bst*EII/*Bcl*I fragment that is immediately at 3′ of the third exon of the NQO2 gene were used to flank a NEO cassette (FRT site-MC1-Neo-FRT site-LoxP site) (as shown in Fig. 1A). A distal LoxP site has been introduced into the unique *Apa*I site located into intron I in order to delete in vitro a region including NQO2 exons 1–3 by Cre-mediated excision.

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Abbreviations: QR1, quinone reductase 1; NQO2, NRH:quinone oxidoreductase 2; BNAH, *N*-benzyldihydronicotinamide; MCA-NAT, 5-methoxycarbonylamino-*N*-acetyltryptamine; NRH, dihydronicotinamide riboside

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.10.083

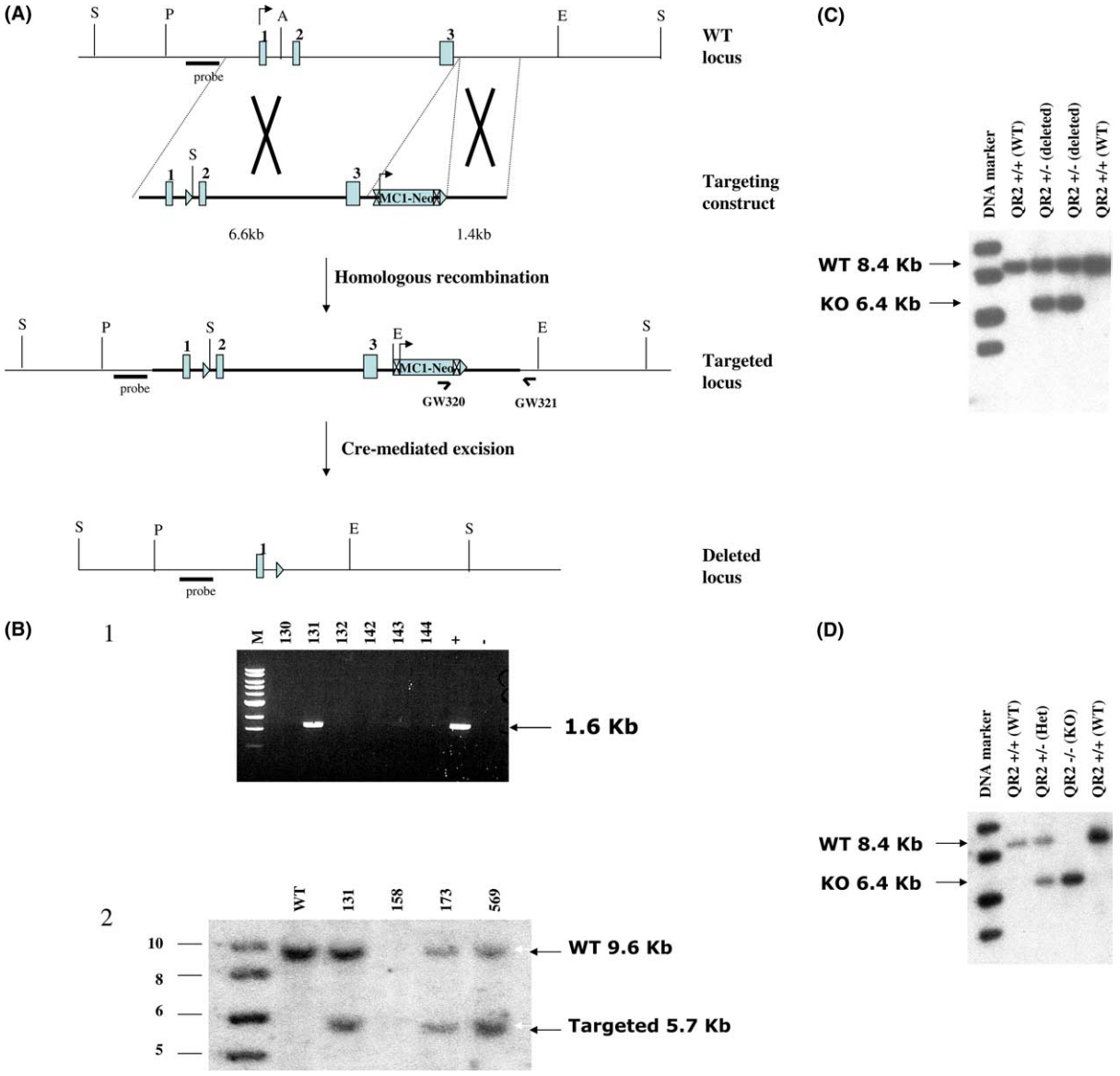

Fig. 1. Targeted disruption of the *NQO2* gene produces a null mutation. (A) Gene targeting strategy for the generation of a NQO2 KO mouse strain. The NQO2 locus (containing the first three exons) and the targeting construct (containing the neomycin (*NEO*) cassette with flanking segments homologous to the locus) are shown in schematic format. The transcriptional orientation of neomycin cassette and the NQO2 locus are delineated by arrows. Note that homologous recombination introduces a LoxP site between exons 1 and 2 and consequently removed the *Apa*I site, while a *Spe*I site has been introduced with the LoxP site. This *Spe*I site is used to screen for positive ES cell clones. The probe used in all the Southern blot analyses is a 1.0-kb fragment located 5′ of the targeting vector. Legend: A: *Apa*I site; E: *Eco*RV site; P: *Pac*I site; S: *Spe*I site; triangle: LoxP site; double triangle: FRT site. (B) Characterization of NQO2 targeted ES cell clones. *Panel 1*: PCR characterization of 3′ targeting of the NQO2 locus. PCR screening on 3′ end of homologous recombination event is shown for ES cell clone #131. The 1.6 kb band signifies the appropriated targeted disruption of m*NQO2* locus. *Panel 2:* Southern blot characterization of NQO2 targeted ES cell clones. Digestion of genomic DNA with *Spe*I resulted in the following diagnostic fragment: wild type allele of 9.6-kb band, appropriate targeted disruption of the NQO2 locus of 5.7-kb band. (C) Southern blot characterization of NQO2 exons 1, 2 and 3 Cre-mediated excised ES cell clones. Digestion of ES cell clones genomic DNA by *Eco*RV/*Pac*I resulted in the following diagnostic fragment: wild type band of 8.4-kb, KO band of 6.4-kb. The absence of 8.4-kb band signified the generation of the NQO2 cells, while a 6.4-kb revealed the presence of a NQO2 KO allele. (D) Southern blot characterization of NQO2 exons 1, 2 and 3 Cre-mediated excised events. Digestion of tail genomic DNA by *Eco*RV/*Pac*I resulted in the following diagnostic fragment: wild type band of 8.4-kb, KO band of 6.4-kb. The absence of 8.4-kb band signified the generation of the NQO2 knockout animal, while a 6.4-kb revealed the presence of a NQO2 KO allele.

2.2. Screening of NQO2 targeted ES cell clones

129SvPas ES cells (genOway SA, Lyon) were electroporated with the linearized targeting construct (40 μg) and selected with G418 (150 μg/ml of active component, Life Technologies, Inc., Gibco-BRL). Homologous recombination in ES cells was assessed via PCR and Southern blot analysis. PCR screening can be described as follows: the GW320 primer was derived from the neomycin cassette (5′-CAGCGCATCGCCTTCTATCG-3′) and the GW321 primer was derived from NQO2 intron 3(5′-GGAAGCGACAGTGACCTACC-3′). PCR conditions were 94 °C/5 min, 35cycles of (94 °C/30 sec, 57 °C/1 min, and 72 °C/2 min) and then 72 °C/10 min, which resulted in a ~1600-bp knockout band. Briefly, for Southern blot analysis, genomic DNA was digested with *Spe*I and then hybridized with a 1.0-kb internal probe; $NQO2^{+/-}$ clones produced a 9.6-kb wild-type band and a

5.7-kb targeted band. Three clones (#131, 173 and 569) were confirmed positive for homologous recombination by both PCR and Southern blot (Fig. 1B).

2.3. Screening for Cre-mediated excision of targeted NQO2 ES cell clones

ES cell clone #131 isolated during the first electroporation was electroporated with a circular Cre-recombinase expression vector. Cre-mediated excision in 100 selected ES clones was assessed by Southern blot analysis. Genomic DNA was digested with *Eco*RV/*Pac*I hybridized with a 1.0-kb internal probe; in order to detect the deletion of NQO2 intron I to intron III. NQO2 KO clones produced a 8.4-kb wild-type and an 6.4-kb Cre-mediated deleted band (Fig. 1C).

2.4. Generation of germ line chimeras and homozygous breeding

One $NQO2^{+/-}$ ES cell clone was microinjected into C57BL/6 blastocysts giving rise to male chimeras with a significant ES cell contribution (as determined by an Agouti coat color). After mating with C57BL/6 females, germ line transmission was confirmed by the genotyping of offspring tail DNA via Southern blot and PCR analysis. F1 male and female heterozygous animals were interbred to obtain NQO2-deficient animals. Homozygous and heterozygous animals were screened by Southern blot analysis as described in the previous section (Fig. 1D).

2.5. Reagents

2-[^{125}I]-MCANAT (specific activity: 2000 Ci/mmol) was custom synthesized by Amersham Pharmacia Biotech (Orsay, France). Menadione were obtained from Sigma (Saint Quentin Favallier, France), whereas *N*-benzyldihydrobenzylnicotinamide (BNAH) was purchased from Maybridge (Cornwell, England). All compounds were dissolved in DMSO at a stock concentration of 10 mM before being stored at −20 °C.

2.6. Biological preparations

Kidneys, and brain of male wild type ($NQO2^{+/+}$), heterozygote ($NQO2^{+/-}$) and knock-out ($NQO2^{-/-}$) mice were obtained frozen at −80 °C from genOway (Lyon, France). All biological sample preparations were performed at 4 °C. Kidneys and brain tissues were thawed and were resuspended in 6 vol of 50 mM Tris/HCl, pH 8.5. Tissues were gently disrupted by 20 strokes of a glass dounce homogenizer (A and B sizes). The homogenates were then centrifuged at $100\,000 \times g$ for 1 h at 4 °C. Supernatants (S1) were saved, while pellets were resuspended in 6 vol of 50 mM Tris/HCl, pH 8.5, using a glass dounce homogenizer (A and B: 2×20 strokes) and centrifuged ($100\,000 \times g$) for 1 h at 4 °C. Both subsequent supernatants (cytosol) and pellets (membrane-rich fraction) were combined and were used for NQO2 enzymatic and 2-[^{125}I]-MCANAT binding assays. Aliquots of all preparations were stored at −80 °C until use. Determination of protein content was performed according to the Bradford method [32] (Bio-Rad SA, Ivry-sur-Seine, France).

2.7. 2-[^{125}I]-MCANAT binding assay

Protein preparations (40–500 μg/ml) were incubated in a 200 μl final volume with 0.2 nM of 2-[^{125}I]-MCANAT (Amersham Pharmacia Biotech) for 15 min at 4 °C in 50 mM Tris/HCl, pH 8.5. Non-specific binding was determined with 10 μM of 2-iodo-MCANAT. The incubation mixture (100 μl) was loaded on a 96 well plate gel filtration method using Sephadex G25 superfine (Amersham Pharmacia, Piscataway, NJ) as chromatographic phase. The gel filtration plate was then centrifuged at 1750 rpm for 25 s at 4 °C and 30 μl of the 2-[^{125}I]-MCANAT bound to proteins was recovered for gamma radioactivity counting. Data were expressed in fmol/mg of protein.

2.8. NQO2 fluorescent enzymatic assay

The oxidoreduction reaction of NQO2 was realized at 25 °C in 200 μl of 50 mM Tris/HCl, pH 8.5, in the presence or not of 100 μM of specific quinone reductase 1 (NQO1) inhibitor, i.e., dicoumarol [33], using 100 μM menadione as substrate and 100 μM of BNAH as co-substrate according to Nosjean et al. [17,21]. Briefly, the decrease of the intrinsic fluorescence of BNAH (excitation at 340 nm with emission at 440 nM in a Polastar 96-well plate reader, BMG, Offenburg, Germany) due to its oxidation in the presence of 100 μM menadione was measured through a kinetic enzymatic method using the FLUOstar Optima software (BMG, Offenburg, Germany). Data were expressed in nmol/min/mg of protein.

2.9. QR1 assay

The QR1 enzymatic activity was measured using 200 μM menadione as substrate and 200 μM of β-nicotinamide adenine dinucleotide in its reduced form (NADH) as co-substrate. The oxidoreduction reaction was realized at 25 °C in 200 μl of 25 mM Tris/HCl and 1 mM *n*-octyl-β-D-glucopyranoside, pH 7.4. The enzymatic kinetic was measured, by following the decrease at 340 nm of the intrinsic fluorescence of the oxidized NADH (Polastar 96-well plate reader, BMG, Offenburg, Germany) according to Zhou et al. [34].

3. Results and discussion

Here, we report the construction of a mouse strain (129sv × C57BL/6) in which the NQO2 gene was invalidated (Fig. 1). Because we want to stabilize the genetic background into a mouse strain having a recognized melatonin rhythm, such as C57BL/6, and because this process will require several months of breeding and crossing, we started with the biochemical characterization of the first KO animals, $NQO2^{-/-}$. We collected several tissues, including brain and kidneys from the homozygous, heterozygous and wild type animals. The genetic analysis of these animals confirmed that they were deprived of the NQO2 gene (Fig. 1). Contrary to what might be assumed, QR1 and NQO2 share similar catalytic properties, in that they are both able to reduce menadione in the presence of BNAH, the stable synthetic NRH analog (F. Vella, G. Ferry and J.A. Boutin, unpublished). Nevertheless, QR1 is highly sensitive to dicoumarol whereas NQO2 is not [23], permitting to differentially characterize their respective activities. The tissue homogenates from the homozygous mice do not support any NQO2 activity, whereas they are able to catalyze the QR1-mediated activities (i.e., menadione reduction in the presence of the co-substrate NADH and sensitive to dicoumarol inhibition), see Table 1.

The 2-[^{125}I]-MCANAT binding on mice organs has been described by Nosjean et al . [17]. It is in the range of a few fmol per mg of protein for mice brain and about 10 times more in the kidney. This situation is similar to what is found in other species, such as hamster and monkey, but not in dog [17]. We measured the same level of binding in similar conditions in the membranes from brain and kidneys from wild-type mice. Table 1 also documents the remarkable loss of melatonin MT_3 binding site using the radioligand 2-[^{125}I]-MCANAT in $NQO_2^{-/-}$ mice.

The IUPHAR compendium for receptors stated that 'A recent report identifies an MT_3 binding site in hamster as the enzyme QR2, as demonstrated by Nosjean et al. 2000]' [10]. Indeed, the present data demonstrate unequivocally that the putative binding site is exclusively this enzyme. No other MT_3 -like melatonin binding site can be detected when the tissues are deprived of NRH-quinone oxidoreductase 2.

Whereas the physiological function of the MT_3 binding site has never been identified, the role of NQO2 is still a debate. Indeed, by analogy with the QR1 enzyme, with which it shares 49% of its amino acid sequence [22], the literature stated that it is a detoxifying enzyme belonging to the Phase II of the drug metabolism and xenobiotic detoxification processes. The data reported on the previous NQO2 knock-out mice by Long

Table 1
Comparison of specific NQO2 enzymatic activity of kidney and brain protein preparations from wild type, heterozygous $NQO2^{+/-}$ and homozygous $NQO2^{-/-}$ mice

	NQO2 enzymatic activity (nmol/min/mg of protein)				Specific binding of 2-[^{125}I]-MCANAT (fmol/mg of protein)		QR1 enzymatic activity (nmol/min/mg of protein)	
	Kidney		Brain		Kidney	Brain	Kidney	Brain
	Dicoumarol (100 μM)		Dicoumarol (100 μM)					
	Without	With	Without	With				
Wild type	14.4 ± 0.7	10.1 ± 0.9	7.0 ± 2.2	6.2 ± 2.0	6.4 ± 1.3	1.5 ± 0.1	2.9 ± 0.1	1.6 ± 0.1
Heterozygotes +/–	12.1 ± 0.1	5.3 ± 0.2	5.3 ± 1.6	4.2 ± 0.6	5.5 ± 0.6	0.5 ± 0.1	3.2 ± 0.2	1.6 ± 0.3
Homozygotes –/–	9.9 ± 4.9	0.3 ± 0.1	1.4 ± 0.8	0.8 ± 0.3	not detected	not detected	2.6 ± 0.1	2.0 ± 0.0

NQO2 catalytic activity was measured using the standard fluorescent assay, with BNAH (100 μM) and menadione (100 μM) as a co-substrate and substrate, respectively. Binding was measured as described [17], using 2-[^{125}I]-MCANAT. QR1 was measured using NADH (200 μM) as co-substrate and menadione as substrate (200 μM) as described [34]. Data were obtained from three different animals for each genotype. Activities were measured at least twice per organ per animal. Results are given as means ± S.D.

et al. [24] clearly showed that it is not the case, and that NQO2 is rather a 'toxifying' enzyme, since the menadione toxicity is dramatically reduced in these knock-out animals [24].

The lack of a natural substrate reported for NQO2 (menadione being a synthetic derivative of vitamin K) and of a natural co-substrate (BNAH being a synthetic derivative of nicotinamide) is perplexing. While we obtained preliminary results strongly suggesting that NQO2 recognizes the natural quinone part of the mitochondrial respiratory chain, Coenzyme Q0, (J.A. Boutin, F. Chatelain-Egger and G. Ferry, submitted for publication), further work will be needed to better understand the role of the enzyme in vivo.

Another perplexing relationship that needs to be documented is the connection between melatonin or 2-iodomelatonin, which have a strong affinity for MT_3 (280 and 8 nM, respectively) in binding studies [17] and a poor inhibition capacity for NQO2 activity (43 and 1 μM, respectively). Although melatonin can be docked inside the catalytic site of NQO2, its poor inhibition capacity remains at least unexpected and still unclear. The fact that NQO2 could be a 'toxifying' enzyme and that melatonin has protective effects suggests that NQO2-inhibition plays an important role. The high concentrations used in vitro required for this protective effect of melatonin [25,26] are compatible with the weak IC_{50} on NQO2. Nevertheless, these concentrations are far from physiology. Indeed, during the night, melatonin level is around 400 pmol/l in plasma and can reach 10 nmol/l in the cerebrospinal fluid, at least in sheep [27]. According to a recent publication [28], melatonin could be produced in lymphocytes at high concentrations, compatible with its documented anti-oxidant capacity at pharmacological concentrations (i.e., in the 100 μM range). At such concentrations, melatonin might inhibit the NQO2-mediated toxifying process towards some of its natural substrates, and therefore, melatonin could preserve some of the activities of lymphocytes or other cellular components, during the burst of oxidative processes seen in the blood during infections and fever events. Protective effects of melatonin have also been reported in different animal models of ischemia [29,30] with pharmacological doses around 10 mg/kg which give plasma levels in rat over 1 μmol/l for at least 30 min [31], therefore in the same range than the inhibition constant for the enzyme.

In the present report, we prove that MT_3 and NQO2 are the same molecular entities, by showing the absence of melatonin binding site and NQO2 activity in tissues issued from NQO2-KO mouse.

Acknowledgements: The authors are grateful to Prof. M.C. Viaud-Massard for the synthesis of 2-iodo-5-methoxycarbonylamino-*N*-acetyltryptamine, to Dr. Roy Golsteyn for his help with the manuscript and to Prof. Alain Ktorza for helpful advices.

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